REMARKS

Claims 11, 13, and 15 -19 are currently pending. Claims 1-10, 12 and 14 were previously canceled. New claim 20 is supported by the pending claims and paragraph [0019] of the application as originally filed.

Claim Rejections under 35 U.S.C. §102

Claim 15 was rejected under 35 U.S.C. § 102(b) as being anticipated by Yamasaki et al (WO 01/47559) using for translation the equivalent Yamasaki et al. (US Patent No. 7018647) as evidenced by Patel et al. (US Patent No. 4855294). In this rejection, glycerin as an anti-irritant is equated to benzocaine as a counter-irritant. A counter-irritant is not the same thing as an anti-irritant. "A **counterirritant** is a substance which creates inflammation in one location with the goal of lessening the inflammation in another location.[1] They can be used as antipruritics. This strategy falls into the more general category of counterstimulation. Capsaicin is an example of a counterirritant.[2]" http://en.wikipedia.org/wiki/Counterirritant.

In contrast, an anti-irritant is defined as "substances that sooth the localized/superficial inflammation of the skin that is due directly to one or more external substances." http://en.mimi.hu/beauty/anti-irritant.html. The Patel et al. reference, USP 4,855,294, uses glycerin to reduce the skin irritation properties of a transdermal drug/permeation enhancer composition. See col. 2, lines 23-28 of the enclosed Patel reference. A compound that reduces irritation can be called an anti-irritant. As noted in the enclosed article (*N. Atrux-Tallau et al.)*, which describe glycerin (glycerol) as a humectant. As a humectant, glycerin is not acting a counter-irritant. Glycerin does not cause a slight inflammation. As defined in the application, "[i]ngredients having a counter-irritation effect, so-called counter-irritants, are agents that cause a slight inflammation upon a topical application to the skin and thereby dissipate the congestion in the tissue below. Counter-irritants are used to alleviate the congestion in the deep

tissue by taking advantage of their ability to stimulate the skin and cause a slight inflammation." See [0005] of the application as filed.

The ingredient having a counter-irritation effect, i.e., counter-irritant, is a medicine applied locally to produce superficial inflammation in order to reduce deeper inflammation; however, the counter-irritant causes unpleasant irritation at the topically applied skin surface. This is nothing like the effect of glycerin (a humectant) on the skin.

The patch taught by the applicants reduces pains, such as muscle pain, joint pain, lumbago, shoulder stiffness, fracture pain and other symptoms associated with pain, without unpleasant irritation when topically applied, while being highly effective. The counter-irritant of the present application shows analgesic and anti-inflammatory effect by producing superficial inflammation in order to reduce deeper inflammation. However, the counter-irritant causes unpleasant irritation, such as pain or stabbing pain. The applicants how found that when benzocaine is also included as an active ingredient, along with an effective amount of a counter-irritant, the result is a reduction in the unpleasant irritation of the counter-irritant, while having a high anti-inflammatory and analgesic effect.

Claim Rejections --35 USC Sec. § 103

Claims 11, 13, and 16-19 were rejected under 35 U.S.C. 103(a) as being unpatentable over Yamasaki et al. (WO 01/47559) using for translation the equivalent Yamasaki et al. (US Patent No. 7018647) in view of Hirashima et al. (US Patent No. 6471984) and as evidenced by Patel et al. (US Patent No. 4855294).

Hirashima et al. relates to a plasticizer for a medicated base for use in a medicated patch. The drugs used in the patch are incidental to description of the plasticizer and its use in Hirashima. The drugs to be used in the patch of Hirashima et al. are not the focus of the disclosure and are presented as a laundry list of anything that could possibly be

used. There is no reason, other than hindsight using the present application, to select benzocaine out of this list. As stated in the Hirashima reference:

> The drug to be used in the patch of this invention is not particularly limited but may be arbitrarily selected from among known conventional drugs.

(Column 2, lines 47-48). Even more telling, methyl salicylate and glycol salicylate are buried in a list of dozens of additives to the hydrophilic base of Hirashima and identified as ultraviolet absorbers or anti-inflammatory agents, not as counter-irritants. As identified as in Hirashima, one skilled in the art would not use these compounds as counter-irritants. No one skilled in the art would have any reason to combine these two random components of the Hirashima et al. patent except by using the present application as a guide and impermissible hindsight to conduct a computer search to select these two items out of the hundreds listed in the Hirashima et al. reference. Moreover, Hirashima does not list the other counter-irritants claimed.

Patel et al. is incorporated in the rejection as an evidential reference in order to verify that glycerin is an anti-irritation agent. Claim 1 of Patel et al. calls for "an effective amount of glycerin to reduce the irritation of said drug-enhancing composition." The abstract of the Patel et al. reference calls "for reducing the skin irritation properties of a transdermal drug/enhancer composition." As noted above, Patel teaches an anti-irritant, a humectant, a soothing compound, glycerin, not a counter-irritant as disclosed and claimed in the present application. Glycerin reduces irritation; it does not cause inflammation, as required for a counter-irritant.

Claims 11, 13 and 16-19 were rejected under 35 U.S.C. 103(a) as being unpatentable over Yamasaki et al. (WO 01/47559) using for translation the equivalent Yamasaki et al. (US Patent No. 7018647) and further in view of Bernstein (US Patent No. 4997853 and as evidenced by Patel et al. (US Patent No. 4855294).

Yamasaki et al and Patel have already been discussed in depth. Bernstein only discloses capsaicin; there is no mention or suggestion of l-menthol, dl-menthol, dl-camphor, d-camphor, methyl salicylate, glycol salicylate, mentha oil, eucalyptus oil, or and nonylic vanillylamide, nor the suggestion of using up to 20 wt% of capsaicin. The largest amount of capsaicin suggested in Bernstein is 1 wt% (see claim 5 and column 1, line 42 of the Bernstein reference).

In particular, new claim 20 claims a range of at least 5% benzocaine up to 15%.

In addition, the focus of the Bernstein reference is to use benzocaine to reduce burning and pain caused by use of capsaicin. There is nothing in Bernstein that would suggest the combination of benzocaine and counter-irritant for non-superficial pain, since Bernstein is directed to "superficial pain syndromes such as postherpetic neuralgia" (See column 1, lines 13-14). The present application is directed to deep muscle, joint and bone pain as noted in paragraph [0001] of the application as filed and claimed in claims 11, 16 and 20.

Applicants respectfully submit that the claims as presented are in condition for allowance.

CONCLUSION

If the Examiner has any questions or suggested Examiner's amendments, the Examiner is respectfully requested to call the undersigned.

The Commissioner is hereby authorized to charge any additional fees, or to credit any overpayment, to Deposit Account No. 50-3195.

Respectfully submitted,

Date: November 29, 2010

/Manette Dennis/
Manette Dennis (Reg. No. 30,623)
Ostrager Chong Flaherty & Broitman, P.C.
570 Lexington Avenue, Floor 17
New York, NY 10022-6894
Tel.: 212 681-0600
Fax: 212 681-0300
mdennis@ocfblaw.com

Appendix

1. N. Atrux-Tallau et al., "Effects of glycerol on human skin damaged by acute sodium lauryl sulphate treatment," Arch. Dermatol. Res. (2010) 302:435-441.

Arch Dermatol Res (2010) 302:435–441
DOI 10.1007/s00403-009-1021-z

ORIGINAL PAPER

Effects of glycerol on human skin damaged by acute sodium lauryl sulphate treatment

Nicolas Atrux-Tallau · Céline Romagny · Karine Padois · Alain Denis · Marek Haftek · Françoise Falson · Fabrice Pirot · Howard I. Maibach

Received: 28 September 2009 / Revised: 15 December 2009 / Accepted: 17 December 2009 / Published online: 31 December 2009

Abstract Glycerol, widely used as humectant, is known to protect against irritants and to accelerate recovery of irritated skin. However, most studies were done with topical formulations (i.e. emulsions) containing glycerol in relatively high amounts, preventing drawing conclusions from direct effects. In this study, acute chemical irritations were performed on the forearm with application of a 10% sodium lauryl sulphate (SLS) aqueous solution under occlusion for 3 h. Then, glycerol aqueous solutions from 1 to 10% were applied under occlusion for 3 h. After elimination of moist excess consecutive to occlusive condition, in ambient air for 15 and 30 min, skin barrier function was investigated by dual measurement of skin hydration and transepidermal water loss (TEWL). Treatments with SLS solution under occlusion significantly increased TEWL and decreased skin hydration as assessed by capacitance measurements. The SLS irritant property was raised by the occlusion and the water barrier function as well as water content appeared impaired. Recovery with glycerol at low doses was remarkable through a mechanism that implies its hygroscopic properties and which is saturable. This precocious effect acts through skin rehydration by enhancing water-holding capacity of stratum corneum that would facilitate the late physiological repair of impaired skin barrier. Thus, glycerol appears to substitute for natural moisturizing factors that have been washed out by the detergent action of SLS, enhancing skin hydration but without restoring skin barrier function as depicted by TEWL values that remained high. Thus, irritant contact dermatitis treated with glycerol application compensate for skin dehydration, favouring physiological process to restore water barrier function of the impaired skin. Empirical use of glycerol added topical formulations onto detergent altered skin was substantiated in the present physicochemical approach.

Keywords Skin · Surfactant · Moisturizer effect · Glycerol · Sodium lauryl sulphate

N. Atrux-Tallau · C. Romagny · K. Padois · F. Falson · F. Pirot (✉)
Laboratoire de Recherche et Développement de Pharmacie Galénique Industrielle, EA 4169, "Fonctions normales et pathologiques de la barrière cutanée", Faculté de Pharmacie, Université Lyon 1, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France
e-mail: fabrice.pirot@recherche.univ-lyon1.fr

A. Denis
Bioderma Laboratoire Dermatologique, 75 Cours Albert Thomas, 69003 Lyon, France

M. Haftek
Laboratoire de Recherche Dermatologique, Pavillon R, EA 4169, "Fonctions normales et pathologiques de la barrière cutanée", Hôpital Edouard Herriot, 5 Place d'Arsonval, 69437 Lyon Cedex 03, France

F. Pirot
Service Pharmaceutique, Pavillon X, Groupement Hospitalier Edouard Herriot, Place d'Arsonval, 69437 Lyon Cedex 03, France

H. I. Maibach
Department of Dermatology, University of California, San Francisco, San Francisco, CA 94143-0989, USA

Introduction

Irritant contact dermatitis is described as a non-immunologic local inflammatory reaction characterized by erythema, oedema, or corrosion following single or repeated application of a chemical substance to an identical

cutaneous site [22]. Irritant contact dermatitis, distinct from allergic contact dermatitis, is caused by excessive contact with irritants without implication of immune reaction. Irritants, include water, soaps, detergents, solvents, acids, alkalis, and friction [6]. SLS is widely used as model irritant to investigate and quantify the effect of single irritant exposure [18]. SLS is an anionic surfactant used in household products and cosmetics that induces skin dermatitis with a high variability amongst persons [21]. Irritant contact dermatitis induced by SLS provokes erythema, dryness, scaling of epidermis leading to skin barrier function alteration [23]. Transepidermal water loss (TEWL) increase after SLS application was described as secondary to stratum corneum hydration due to spongiosis (inflammatory intercellular edema of the epidermis) [19]. Those modifications in TEWL and hydration reveal a skin barrier impairment supported by the stratum corneum. In normal SC, it is thought that the ratio of lipids in ordered (e.g. orthorhombic and hexagonal) and disordered (e.g. liquid crystalline) phases [11] modulate the SC barrier function properties. Thus, organization of lipid bilayers rather than the extraction of lipids is responsible for barrier impairment [19, 28]. Denaturation of α-helical keratin proteins due to conformational interaction with surfactants may be implicated in increased water absorption leading to surfactant-induced stratum corneum swelling [23]. In order to maintain its flexibility and integrity, the stratum corneum must remain hydrated, and in healthy skin, the water content is about 30 wt.% [27]. In the absence of water, the stratum corneum is an intrinsically fragile structure that readily becomes cracked, brittle, and rigid. Glycerol represents a hygroscopic compound capable of absorbing water from the environment and deeper parts of the stratum corneum. It is one of the best natural moisturizers in living systems and has been used in skin care products for preventing and treating skin dryness because it moisturizes/plasticizes the stratum corneum. Glycerol is naturally present in skin catabolic product of sebaceous gland-derived triglycerides and is also transported from dermis to basal layers of the skin through aquaporine 3 (AQP3) transmembrane transporting protein. A defect in AQP3 in mice is correlated to a decrease in glycerol content in skin and a reduced stratum corneum hydration [14]. Until recently, the moisturizing benefits were attributed to its humectant action; however, diglycerol and triglycerol with higher humectant activity demonstrated low improvement in skin dryness as compared to glycerol in guinea pig model [14]. It is now known that the skin care benefits of glycerine include attraction of moisture, osmoregulation of the intracellular milieu, maintenance of crystallinity/fluidity of cell membranes and intercellular lipids [20]. Andersen studied antiirritants effect on acute and cumulative irritation induced by SLS or nonanoic acid applications in hairless guinea pig model and in human volunteers in its extensive studies; only glycerol treatments improved skin barrier function parameters after irritation [2–5]. Glycerol improvement in skin barrier recovery following mechanical (i.e. tape stripping) or chemical (i.e. repeated SLS application) damage was assumed to act through a stimulus for barrier repair and not only through humectant properties; thus, glycerol can be regarded as a barrier stabilizing and moisturizing compound [15].

The present study characterizes the effect of glycerol onto skin damaged by SLS occlusive exposure. Chemical or physical damages of skin barrier function, mainly supported by stratum corneum, is classically assessed by TEWL and hydration measurements [7]. Thus, potential beneficial effects of glycerol on skin barrier function disrupted by SLS were investigated by dual measurement of TEWL and hydration to clarify mechanisms of glycerol onto water transport through chemically damaged stratum corneum.

Materials and methods

Biophysical parameters of normal skin

Four healthy informed human female volunteers from 24 to 28 years old were included in topical treatment experiment. The exclusion criteria were the following: pregnancy or lactation; risk of poor cooperation; any dermatological disorders, ongoing pharmaceutical treatment, history or clinical signs of atopic dermatitis or contact dermatitis. Any procedure (e.g. topical application of SLS) was not considered as invasive. The age of the tested group guaranteed a good response to SLS-induced irritation as SLS irritancy sensitivity was suggested to decrease in the elderly [12, 13, 21].

Seven skin areas (2×2 cm) were delimited, with a dermatological pen, on the volar forearm surface at 10 cm from the wrist, in avoiding hairy zone. Basal values of TEWL and hydration were performed with a Vapometer (Delfin Technologies Ltd, Finland) and a Corneometer CH 825 PC (Courage and Khazaka, Köln, Germany), respectively. Recordings were performed in a laboratory at 22 ± 2°C with constant relative humidity (60 ± 5%). The inter-individual variance was found negligible concerning TEWL basal values (ANOVA test, 4 groups, 27 DF, $P > 0.05$) supporting that the skin barrier properties were found homogeneously distributed in volunteer group. However, basal hydration exhibited statistical differences in volunteers (ANOVA test, 4 groups, 27 DF, $P < 0.001$) disclosing the intrinsic complexity of hydration phenomenon.

Biophysical parameters of skin after acute chemical irritation (ACI) by SLS

Six of the delimited sites were treated for 3 h with polypropylene plastic closed chamber (18 mm diameter, 254 mm^2, Hill Top®, Cincinnati, USA) filled with 200 µL of 10% SLS aqueous solution (98.5% purity, Sigma, St. Louis, USA). One site was treated with 200 µL deionised water as control site. At the end of the 3-h period, the chambers were removed and skin areas were gently dried with cotton swab. After 15 min, SLS and water-treated sites were measured for TEWL and hydration.

Biophysical parameters of skin after ACI by SLS and post glycerol treatment

Sodium lauryl sulphate (SLS) and water pre-treated skin areas were exposed to 200 µL of glycerol solutions (99.8% purity, Sigma, St. Louis, USA) at 0–10% (v/v). The previous control skin area was treated with 200 µL of deionised water. After 3 h, all chambers were removed and skin areas were gently dried with cotton swab. After 15 and 30 min, TEWL and hydration measurements were performed.

Results

Biophysical parameters of normal skin and after ACI by SLS

Transepidermal water loss is restricted by intact stratum corneum in normal healthy skin. Upon all 28 sites tested (4 volunteers, 7 sites each) before treatments, basal TEWL values measured were 5.9 ± 0.7 g m^{-2} h^{-1}. Acute cutaneous exposure to highly concentrated SLS aqueous solution in occlusive conditions for 3 h raised significantly TEWL up to 21.4 ± 8.1 g m^{-2} h^{-1} ($n = 24$) ($P < 0.001$ as compared to basal values). The combined effects of pure occlusion and proper SLS irritant properties upon skin barrier properties might be deconvoluted as follows:

$$1 - \frac{[\mathrm{TEWL_{water}} - \mathrm{TEWL_{SLS}}]}{\mathrm{TEWL_{water}}}. \quad (1)$$

$TEWL_{water}$ and $TEWL_{SLS}$ were determined on 3-h water and SLS aqueous solution-treated skin areas (17.1 ± 2.7 g m^{-2} h^{-1}, $n = 4$ and 21.4 ± 8.1 g m^{-2} h^{-1}, $n = 24$ respectively). Clearly, occlusion counted for ~80% of the increase of water flux through the stratum corneum. Consequently, synergistic or additive interaction of SLS represented only 20% of total extent of TEWL, so that no difference was shown between TEWL values on SLS and water-treated skin under occlusion conditions (Fig. 1).

Hydration levels were comparable between basal and water-treated skin areas ($P > 0.05$) (Fig. 1). However, hydration levels on SLS-treated skin areas was significantly lower than those determined in untreated (basal values) and water-treated skin areas ($P < 0.001$; 37.5 ± 8.2 AU vs. 61.1 ± 9.7 AU and vs. 63.8 ± 10.8 AU, respectively). Therefore, deleterious effect of SLS acute treatment onto water-holding properties of skin was confirmed while perturbations of skin barrier properties assessed by TEWL measurements were found minimal.

Biophysical parameters of skin after ACI by SLS and post-glycerol treatment

Beneficial effects of glycerol aqueous solutions onto the recovery of skin barrier function after ACI were evaluated by varying glycerol concentration and subsequent biophysical parameter measurements. Figure 2a showed that the TEWL was not restored to basal values after ACI and 1–10% glycerol post-treatment. Clearly, ACI was sustained, whereas SLS was removed. Consequently, the beneficial effects of glycerol onto transepidermal water rate were discreet for small concentrations ranged between 1 and 2%. Although, ACI was persistent despite glycerol treatment, its extent was reduced by exogenous application of high concentrations of glycerol (3–10%). To evidence glycerol concentration effects upon TEWL recovery onto ACI sites, data were grouped as values obtained from 0, 1 and 2% glycerol solutions (0–2% glycerol group), and from 3, 5 and 10% glycerol solutions (3–10% glycerol group) (Fig. 2b). After 15 min of 3-h glycerol post-ACI, significant differences were found in TEWL values between 0 and 2%

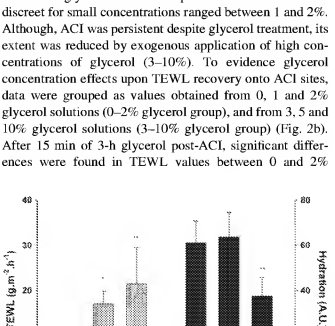

Fig. 1 Transepidermal water loss (TEWL) and skin hydration measured onto (1) normal skin (basal values, $n = 28$) (2) water ($n = 4$) and (3) SLS ($n = 24$)-treated skin under occlusion for 3 h. After 3-h occlusion, water and SLS treatments increased significantly (*$P < 0.01$), whereas a significant reduction in cutaneous hydration was noted onto SLS-treated skin (**$P < 0.001$). Each data are presented as mean ± standard deviation

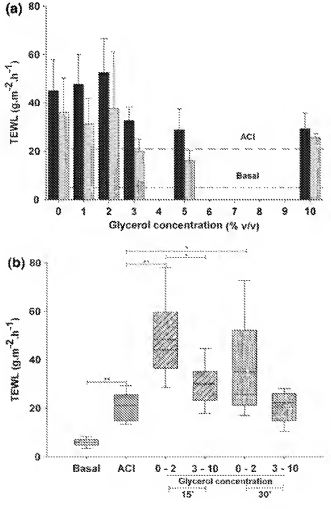

Fig. 2 **a** Transepidermal water loss 15 min (*black bars*) and 30 min (*gray bars*) after the 3-h recovery period under occluded chambers as a function of glycerol concentrations. *Dashed line* specifies mean TEWL at basal state and *dashed-dotted line* specifies TEWL values after ACI. It appears that solutions above 2% glycerol decrease TEWL suggesting a beneficial effect on barrier recovery. Data are presented as the mean value ± SEM of four sites (ANOVA test, $*P < 0.05$, $**P < 0.01$ as compared to 0% glycerol group). **b** Box plots of TEWL values grouped as basal values (*gray boxes*, $n = 28$), ACI sites (*dark gray* boxes, $n = 24$), 0–2% glycerol and 3–10% glycerol groups measured after 15 min (*striped boxes*, $n = 12$) or 30 min (*dotted boxes*, $n = 12$) from the end of the 3-h recovery time. The results are presented as box plots with median values (*solid lines*) and mean values (*dashed lines*). The *bottom boundary* of the box indicates the 25th percentile and the *top boundary* indicates the 75th percentile. *Whiskers* (*error bars*) above and below the box indicate the 90th and 10th percentiles (Student *t* test, $**P < 0.001$, $*P < 0.01$)

glycerol group (TEWL 48.4 ± 16.3 g m^{-2} h^{-1}), 3–10% glycerol group (30.3 ± 8.6 g m^{-2} h^{-1}) as compared to ACI sites (17.7 ± 1.6 g m^{-2} h^{-1}) (Fig. 2b). In addition, 30 min after 3-h glycerol post-ACI, statistical differences were found between 0 and 2% glycerol group and ACI site, suggesting that glycerol treatment above 3% glycerol restrained irritation induced by SLS while solutions below 3% glycerol did not abolish extent of TEWL after ACI (Fig. 2b). Overall, as depicted in Fig. 2, an increase or persistence of high water fluxes through skin presenting ACI was noted for smallest (0–2%) and highest (3–10%) glycerol concentrations, respectively. Figure 3a showed that the hydration after glycerol treatment post-ACI was either restored or increased when compared with basal values. Hydration onto ACI sites was improved as a function of glycerol concentration reaching a plateau above 2% glycerol concentration (Fig. 3a). Transient increase in hydration onto water-treated site (57.3 ± 12.2 AU at 15 min and 48.0 ± 7.2 AU at 30 min) was higher than that from ACI sites, but lower than that from basal values. Hydration recovery consecutive to glycerol treatment improved hydration status as a function of glycerol concentration (Fig. 3b). A significant higher hydration was found between 0 and 1% glycerol group and 2 and 10% group ($P < 0.01$ and $P < 0.001$ after 15-min and 30-min readings, respectively). Overall, it appeared that glycerol treatment above 2% *v/v* restored skin hydration to a level higher than basal sites (Fig. 3b). Increasing glycerol concentration in ACI sites decreased TEWL values and increased hydration values with a plateau phase. Taking into consideration that skin barrier function of the skin was not restored in such a short time exposure (e.g. 3 h), the effects of glycerol on the skin biophysical parameters measured were likely due to hygroscopic properties mimicking natural moisturizing factors (NMF) responsible for the water-holding capacity (WHC) of stratum corneum. Considering that WHC reflected an equilibrium between bound and free water determined, respectively, by hydration (H) measurements and TEWL, the variation of WHC (ΔWHC) induced by glycerol treatment was determined as followed:

$$\Delta \mathrm{WHC}_{\mathrm{Glycerol}}(\%) = 100\left[\left(\frac{H_{\mathrm{Glycerol}}}{H_{\mathrm{Water}}}\right) - \left(\frac{\mathrm{TEWL}_{\mathrm{Glycerol}}}{\mathrm{TEWL}_{\mathrm{Water}}}\right)\right], \quad (2)$$

where H_{Glycerol}, H_{Water}, $\mathrm{TEWL}_{\mathrm{Glycerol}}$ and $\mathrm{TEWL}_{\mathrm{Water}}$ were measured on sites treated with or without glycerol, respectively, for 3 h under occlusion after ACI induction. Figure 4 depicts the variations of WHC of the skin as a function of glycerol concentrations applied on ACI sites. It appears that glycerol improved drastically WHC of the skin from concentrations as low as 1%, whereas above 3% a plateau phase exists suggesting that the skin is saturated with bound water.

Discussion

Classically, TEWL measurement is used to assess skin water barrier function and is proposed as an endogenous standard for permeation studies [8] while stratum corneum

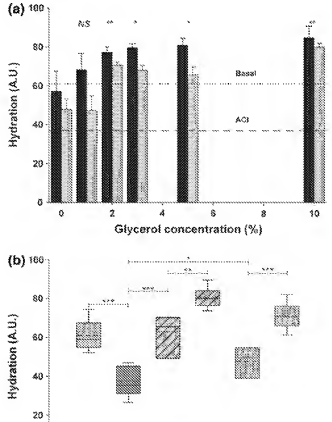

Fig. 3 **a** Skin hydration at 15 min (*black bars*) and 30 min (*gray bars*) after the 3-h recovery period under occluded chambers as a function of glycerol concentrations. *Dashed line* specifies mean hydration at basal state and *dashed-dotted line* specifies hydration values after ACI. Data show an increase in hydration as a function of glycerol content with a plateau phase starting with the 2% glycerol solution. Each point is the mean value ± SEM of four sites. **b** Box plots of hydration values grouped as basal values (*gray boxes*, $n = 28$), SLS-treated sites (*dark gray boxes*, $n = 24$), 0–1% glycerol (*striped boxes*, $n = 8$) and 2–10% glycerol (*dotted boxes*, $n = 16$); measurements were realized after 15 or 30 min from the end of the 3-h recovery time. The results are presented as box plots with median values (*solid lines*) and mean values (*dashed lines*). The *bottom boundary* of the box indicates the 25th percentile and the *top boundary* indicates the 75th percentile. *Whiskers* (*error bars*) above and below the box indicate the 90th and 10th percentiles; 15 min after the 3-h recovery period hydration increased significantly in all sites as compared to data after SLS treatment, but only solutions above 2% glycerol enhanced hydration significantly as compared to basal values. Moreover, 30 min after recovery, only solutions above 2% glycerol presented a hydration value greater than basal values. Student *t* test analysis was realized after testing the standard deviation of populations involved; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

capacitance reflects the hydration of the skin. However, these parameters are linked: TEWL is described as a function of Fick's first law of diffusion (Eq. 3)

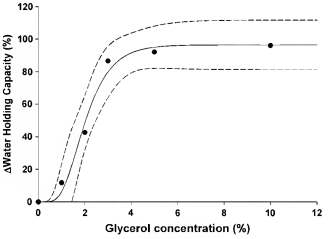

Fig. 4 Water-holding capacity of the skin as a function of glycerol concentration (*v*/*v*%) used after ACI. *Solid line* represents the sigmoidal regression of the plot (Chapman, 3 parameters, $R^2 = 0.987$) and *dashed lines* delimit the 95% confidence band. Increasing glycerol concentration on irritated skin increases water content of the skin with a plateau phase of action

$$\mathrm{TEWL} = \frac{K\bar{D}}{H}\Delta C = K_p \Delta C, \tag{3}$$

where K is the partition coefficient of water between SC and viable epidermis; D is the average apparent diffusivity of water in the SC of thickness, H (μm); and ΔC is the water concentration difference across the membrane (e.g. concentration in the SC minus concentration above SC). K_p is called the permeability coefficient of water across the SC [17].

In vivo testing of alkyl sulfates was reported to induce hyper-hydration of the stratum corneum and irritancy. This was noticed after a short exposure (e.g. 5 min) under occluded chamber and was rapidly reversible. SLS was presented as the alkyl sulfate giving the higher hyper-hydration of the stratum corneum and upper irritation potency at short time exposure. This hyper-hydration was followed by sub-basal values of hydration and explained by the SC structure disorganization leading to over exposed water-binding sites facilitating water fixation in a first part and lability thereafter leading to skin dryness [28]. The design of this experiment did not point out the hyper-hydration effect following SLS exposure with a general decrease in hydration of 38.6%. The disruption of the barrier function was assessed with a four time increase in TEWL following SLS treatment. However, this increase may not be totally attributed to SLS irritancy because a significant increase in TEWL value was reported following occlusion with water about three time basal values. This effect was due to occlusion itself and water irritancy which was reported to disrupt temporarily the SC [1, 16]. Moreover, the 3-h period SLS treatment may have washed

out water-binding moisturizers leading to enhanced dehydration.

Skin barrier recovery, after SLS-induced irritation, with increasing amounts of glycerol was assayed following TEWL and SC hydration. Glycerol is a well-known moisturizer and belongs to the NMF. It has a poor conductivity (i.e. 0.064 μS cm^{-1}, at 25°C) thus may not interfere with the capacitance measurement (e.g. SC hydration). Glycerol pre-treatment diminish DMSO, NaOH and SLS irritancy and several publications reported faster recovery with glycerol after physical (e.g. tape stripping) or chemical (e.g. acetone, SLS) treatments in various conditions [14]. This effect was suggested to act through the high hygroscopicity of glycerol, supporting TEWL and ion, especially calcium, movements; however, no study in human skin permitted to confirm this hypothesis [10]. Most of the studies were done with relatively high amount of glycerol (e.g. from 5% up to 40%) often included in emulsions or cream. In the present study, we experienced aqueous solution of glycerol from 1 to 10% *v*/*v* to evaluate the minimal effective glycerol concentration.

Hydration was restored to basal values with solutions of 2% glycerol or more which was not linearly correlated to the concentration as 2, 3 and 5% glycerol solutions induced similar response to hydration and 10% solution a better hydration 30 min after 3-h recovery (Fig. 3a). The observed improvement of skin hydration is not supported by occlusion as untreated site (e.g. 0% glycerol) did not permit recovery to basal values. Thus, it appears that glycerol improve hydration for concentration as little as 2% *v*/*v*, which is ten times higher than endogenous glycerol content in the forearm skin estimated at 0.2 μg/cm^2 [29]. Water uptake by glycerol embedded in extra and intracellular domains of SC elevated transiently skin hydration by osmosis (i.e. displacement of free water towards highly concentrated compartment) which might be appreciated by the elevation of TEWL. Glycerol application did not permit restoration of barrier function after 3-h occlusion as suggested by TEWL values which were still significantly higher than basal values (Fig. 2b). We observed TEWL values higher than post-ACI suggesting that (1) remaining SLS continue to disturb SC lipid organization (2) supplementary 3 h occlusion after ACI-enhanced irritation. Albeit glycerol application of low concentrations (e.g. 1 and 2% solutions) did not counterbalance this situation (Fig. 2a), the application of 3% and above glycerol solution restrained TEWL values to post-ACI values. The application of a sufficient dose of glycerol may cover uniformly the skin, modifying the water partition coefficient and thus TEWL (Eq. 3). In addition, it has been reported that glycerol facilitated skin penetration of topically applied drugs [9, 15] which was suggested to act through the increase in SC hydration and in some parts by subtle changes in the lipid organization through an inhibition of the transition from the liquid crystalline form to solid crystalline form.

From these observations, it appears that glycerol efficacy on hydration and TEWL reached a plateau phase when enhancing glycerol concentration, resulting in a maximal WHC value. A similar result was recorded when a kinetic approach of WHC as a function of glycerol concentration was realized [24]. WHC of stratum corneum has been related to hygroscopic compounds (e.g. NMF such as amino acids, urea…) and particularly to SC osmotic pressure which relies on (1) exogenous pressure (i.e., molar concentration) and (2) the SC permeability to compounds [24–26]. Hence, glycerol may achieve maximal solubility in the lipid-rich SC leading to maximal WHC as depicted by the plateau phase (Fig. 4). Consequently, topical preparations containing low doses of glycerol (i.e. 5% or less) may be preferred to high-glycerol content that may induce dehydration of viable epidermis through osmotic pressure.

Conclusion

Glycerol is widely used in pharmaceutical formulations and cosmetics for its' good emollient and humectant properties due to hygroscopicity. It is usually regarded as a nontoxic and nonirritant material. In this study, the potential beneficial effect of glycerol on skin barrier function disrupted by ACI acts through the increase in WHC. This was observed for glycerol concentrations as little as 2% followed by a plateau phase above 5% suggesting a saturation of the system. Glycerol did not counteract SLS consequences on SC, but improving water content of the skin would help physiological repair of the impaired barrier. Thus, constitutive skin dehydration (e.g. ichtyosis) and casual dry skin (e.g. seasonal cold dry weather, pharmacological treatments) may be treated with low-dose glycerol giving maximal efficacy and good patient requirement compliance. Clinical care of including glycerol in cosmetic and medicinal skin formulations was, therefore, scientifically justified in this physicochemical approach.

Acknowledgments N. Atrux-Tallau was granted by Bioderma Laboratoire Dermatologique, Lyon, France, and the French Ministry for Education and Research is acknowledged for their financial support (CIFRE agreement 1169/2006).

References

1. Agner T, Serup J (1993) Time course of occlusive effects on skin evaluated by measurement of transepidermal water loss (TEWL): including patch tests with sodium lauryl sulphate and water. Contact Dermatitis 28(1):6–9

2. Andersen F, Hedegaard K, Fullerton A, Petersen TK, Bindslev-Jensen C, Andersen KE (2006) The hairless guinea-pig as a model for treatment of acute irritation in humans. Skin Res Technol 12(3):183–189
3. Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE (2006) Anti-irritants I: dose–response in acute irritation. Contact Dermatitis 55(3):148–154
4. Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE (2006) Anti-irritants II: efficacy against cumulative irritation. Contact Dermatitis 55(3):155–159
5. Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE (2006) The hairless guinea-pig as a model for treatment of cumulative irritation in humans. Skin Res Technol 12(1):60–67
6. Anonymous (1997) Allergic contact dermatitis, contact allergy. In: Eczema, dermatitis and allergies. New Zealand Dermatological Society Incorporated. http://www.dermnetnz.org/dermatitis/contact-allergy.html. Accessed 15 Jan 2008
7. Atrux-Tallau N, Huynh NT, Gardette L, Pailler-Mattei C, Zahouani H, Viviant E, Hirsch H, Haftek M, Falson F, Pirot F (2008) Effects of physical and chemical treatments upon biophysical properties and micro-relief of human skin. Arch Dermatol Res 300(5):243–251
8. Atrux-Tallau N, Pirot F, Falson F, Roberts MS, Maibach HI (2007) Qualitative and quantitative comparison of heat separated epidermis and dermatomed skin in percutaneous absorption studies. Arch Dermatol Res 299(10):507–511
9. Barichello JM, Yamakawa N, Kisyuku M, Handa H, Shibata T, Ishida T, Kiwada H (2008) Combined effect of liposomalization and addition of glycerol on the transdermal delivery of isosorbide 5-nitrate in rat skin. Int J Pharm 357(1–2):199–205
10. Bettinger J, Gloor M, Peter C, Kleesz P, Fluhr J, Gehring W (1998) Opposing effects of glycerol on the protective function of the horny layer against irritants and on the penetration of hexyl nicotinate. Dermatology 197(1):18–24
11. Boncheva M, Damien F, Normand V (2008) Molecular organization of the lipid matrix in intact Stratum corneum using ATR-FTIR spectroscopy. Biochim Biophys Acta 1778(5):1344–1355
12. Cua AB, Wilhelm KP, Maibach HI (1990) Cutaneous sodium lauryl sulphate irritation potential: age and regional variability. Br J Dermatol 123(5):607–613
13. Elsner P, Wilhelm D, Maibach HI (1990) Irritant effect of a model surfactant on the human vulva and forearm: age-related differences. J Reprod Med 35(11):1035–1039
14. Fluhr JW, Darlenski R, Surber C (2008) Glycerol and the skin: holistic approach to its origin and functions. Br J Dermatol 159(1):23–34
15. Fluhr JW, Gloor M, Lehmann L, Lazzerini S, Distante F, Berardesca E (1999) Glycerol accelerates recovery of barrier function in vivo. Acta Derm Venereol 79(6):418–421
16. Friebe K, Effendy I, Loffler H (2003) Effects of skin occlusion in patch testing with sodium lauryl sulphate. Br J Dermatol 148(1):65–69
17. Kalia YN, Pirot F, Guy RH (1996) Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum in vivo. Biophys J 71(5):2692–2700
18. Kartono F, Maibach HI (2006) Irritants in combination with a synergistic or additive effect on the skin response: an overview of tandem irritation studies. Contact Dermatitis 54(6):303–312
19. Lévêque JL, De Rigal J, Saint-Léger D, Billy D (1993) How does sodium lauryl sulfate alter the skin barrier function in man? A multiparametric approach. Skin Pharmacol 6(2):111–115
20. Lodén M, Maibach HI (2000) Dry skin and moisturizers: chemistry and function. CRC Press, Boca Raton
21. Marrakchi S, Maibach HI (2006) Sodium lauryl sulfate-induced irritation in the human face: regional and age-related differences. Skin Pharmacol Physiol 19(3):177–180
22. Mathias CG, Maibach HI (1978) Dermatotoxicology monographs I: cutaneous irritation: factors influencing the response to irritants. Clin Toxicol 13(3):333–346
23. Patil SM, Singh P, Maibach HI (1994) Cumulative irritancy in man to sodium lauryl sulfate: the overlap phenomenon. Int J Pharm 110(2):147–154
24. Pirot F, Falson F, Pailler-Mattei C, Maibach HI (2006) Stratum corneum: an ideal osmometer? Exog Dermatol 3(6):339–349
25. Pirot F, Morel B, Peyrot G, Vuillet T, Faivre V, Bodeau C, Falson F (2003) Effects of osmosis on water-holding capacity of stratum corneum and skin hydration. Exog Dermatol 2(5):252–257
26. Potts RO, Guy RH (1992) Predicting skin permeability. Pharm Res 9(5):663–669
27. Silva CL, Topgaard D, Kocherbitov V, Sousa JJS, Pais AACC, Sparr E (2007) Stratum corneum hydration: phase transformations and mobility in stratum corneum, extracted lipids and isolated corneocytes. Biochim Biophys Acta Biomembr 1768(11):2647–2659
28. Wilhelm KP, Cua AB, Wolff HH, Maibach HI (1993) Surfactant-induced stratum corneum hydration in vivo: prediction of the irritation potential of anionic surfactants. J Invest Dermatol 101(3):310–315
29. Yoneya T, Nishijima Y (1979) Determination of free glycerol on human skin surface. Biomed Mass Spectrom 6(5):191–193